Fungal Growth Stimulation by CO_2 and Root Exudates in Vesicular-Arbuscular Mycorrhizal Symbiosis

Transformed roots of carrot were used to determine the effects of root metabolites on hyphal development from spores of the vesicular-arbuscular mycorrhizal fungus *Gigaspora margarita*. Hyphal growth of this obligately biotrophic symbiont was greatly stimulated by a synergistic interaction between volatile and exudated factors produced by roots. Root volatiles alone provided little stimulation, and root exudates alone had no effect. For the first time, carbon dioxide was demonstrated to be a critical root volatile involved in the enhancement of hyphal growth. ^{14}C-labeled root volatiles were fixed by the fungus and thus strongly suggested that CO_2 served as an essential carbon source.

Spores of most species of vesicular-arbuscular mycorrhizal (VAM) fungi can readily germinate in vitro, but subsequent hyphal development is limited. This hyphal growth continues over a relatively short time interval and ceases before total depletion of spore reserves. It has sometimes been increased under different nutritional conditions, but unlimited hyphal elongation has never been obtained. The mechanisms determining this hyphal growth inhibition are not yet known, and they may be the reasons for our failures in maintaining pure cultures of VAM fungi. Attempts to discover the factors which can overcome this inhibition are very important from both the fundamental and applied viewpoints. Different hypotheses (16) have considered nutritional, physical, and genetic factors, but none of them has been clearly demonstrated.

Using an in vitro system to interactively grow transformed roots and *Gigaspora margarita* Becker & Hall on a common medium, Bécard and Piché (3a) distinguished two mechanisms by which the roots contribute to fungal growth. The first mechanism (M1) is triggered immediately by the presence of roots and is responsible for an important stimulation of hyphal growth from a germinated spore. This growth still requires the presence of the spore, and it ceases progressively, presumably because of the progressive depletion of spore reserves. The second mechanism (M2), independent of the presence of the spore, is established when the first arbuscules are forming. Therefore, the two mechanisms are differentiated by the nutritional source for the hyphae: the spore for M1 and the host for M2.

The activation of M1 occurs before fungal contact with the roots. Since fungal growth stops immediately after the removal of roots, Bécard and Piché (3a) suggested that an alteration of gaseous components could be involved. The present study is a more thorough investigation of the root factors involved in the induction of fungal growth. By manipulating the dual culture system described by Bécard and Piché (3a), we measured hyphal elongation under the influence of volatile compounds and exudates produced by growing roots. This integrated approach allowed us to demonstrate the critical role that carbon dioxide plays in fungal growth.

MATERIALS AND METHODS

Root organ culture. A clone of root-inducing-T-DNA-transformed root of carrot, established by Bécard and Fortin (3), was routinely propagated on a minimal (M) medium (3) in petri dishes. For use as the plant partner in interactions with the fungus, root explants were standardized and prepared as described by Bécard and Piché (3a). These root explants have vigorous elongation zones and a characteristic pattern of lateral root formation.

Fungal inoculum. Spores of *G. margarita* Becker & Hall (DAOM 194757; deposited at the Biosystematic Research Center, Ottawa, Ontario, Canada) were produced, collected, surface sterilized, stored, and used as the fungal inoculum as previously described (3).

General conditions for fungal growth. A single spore per petri dish was used as an experimental unit for all experiments. Dishes were sealed thoroughly with Parafilm to confine the internal atmosphere and incubated in the dark at 26°C. They were stood up vertically so that the germ tube of *G. margarita* spores would elongate upward as a result of its negatively geotropic mode of growth (31). Cotton rolls (dental rolls; Healthco DDL, Montreal, Quebec, Canada) were placed in petri dishes to absorb excess water. Hyphae from germinating spores were grown either on fresh M medium or on M medium containing root exudates (ME medium). ME medium was prepared by cultivating two root explants on fresh M medium for 2 weeks. These roots were grown on a thin layer of M medium covering a dialysis membrane (molecular weight cutoff, 12,000 to 14,000; Spectra/por 2; Spectrum Medical Industries, Inc., Los Angeles, Calif.) which lay on the bulk of the agar medium. In this way, the roots produced exudates which diffused through the membrane into the agar medium, and their subsequent removal with the membrane was greatly facilitated. Before use, the dialysis membrane had been washed for 24 h in running distilled water and autoclaved at 121°C for 15 min.

Carbon dioxide was removed from the gaseous phase of selected cultures with KOH traps. Each KOH trap consisted of 3 ml of 1 M KOH on two cotton dentals rolls fitted into a small uncovered petri dish (35 by 10 mm). Two traps were added to selected experimental units by being inserted into circular holes of corresponding sizes made in the agar. These operations were all made axenically. All experiments with KOH traps had corresponding control treatments in which

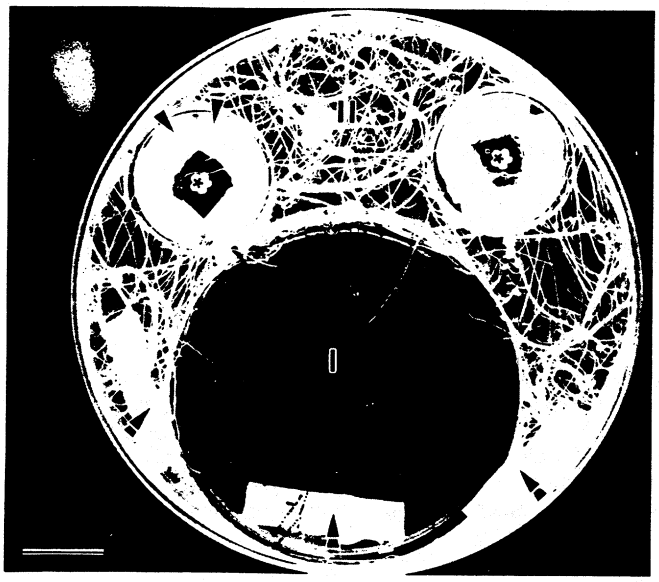

FIG. 1. The bicompartmental system used to study the influence of root volatiles on fungal growth. One germinating spore of *Gigaspora margarita* is placed on M or ME medium in compartment I, the dots tracing hyphal elongation. Roots are grown on M medium in compartment II. Each of the two KOH traps (*) contains two cotton rolls (arrowheads) soaked in KOH. Three cotton rolls (double arrowheads) at the bottom are used to absorb excess water. Bar, 2 cm.

KOH was replaced by water. CO_2 concentrations were checked occasionally in the different culture systems by analyzing gas samples (1 ml) with a gas-partitioner (Fisher-Hamilton). The gas sample was collected from sealed petri dishes by inserting a syringe needle (20-gauge, 1.5 in., Yale; Becton-Dickinson Canada, Mississauga, Ontario, Canada), which had been bent to form a 90° angle, through the Parafilm and between the cover and the bottom of the dish.

Fungal growth in presence of roots. (i) Bicompartmental system. Two compartments were created by placing an uncovered petri dish (100 by 15 mm) into a large petri dish (150 by 25 mm). It was therefore possible to expose a fungal culture to gaseous conditions in the absence of growing roots or to gaseous conditions modified by the presence of roots growing in a separate compartment. The fungus was always cultured on medium in the small dish. Treatments with roots used three root explants, and in some cases, they were modified by adding two KOH traps (Fig. 1). Conditions in which the fungus grew on M and ME medium in the presence of root volatiles are referred to as MV and MEV, respectively.

(ii) Common medium. In square petri dishes (100 by 15 mm), the spore and root were grown on a common medium to achieve a dual culture in which root contacts and infections by hyphae occur (3a). Eight days after the first contact between the germ tube and the root, the germ tube was severed close to the spore and the spore was removed from the dual culture. These two operations were made simultaneously with a red-hot scalpel as previously described (3a). Three days later, gaseous CO_2 in selected dual cultures was removed by replacing the cover of the petri dish with the bottom of another petri dish holding KOH traps. The pair of petri dishes was held together with Parafilm.

Fungal growth under controlled atmospheric conditions. Three days after spore germination, hyphal elongation was

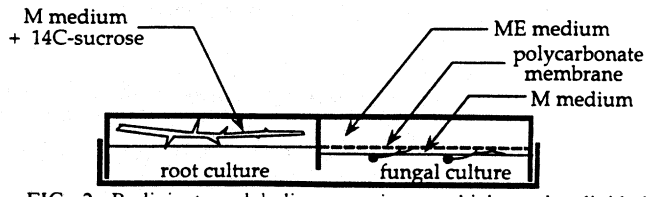

FIG. 2. Radioisotope labeling experiment which used a divided petri dish to show the transfer of volatile root metabolites to the fungal culture.

left to continue for 3 weeks under atmospheric conditions provided by cylinders of compressed gas: atmospheric air (0.03% CO_2; Canadian Liquid Air Ltd.) or atmospheric air supplemented with CO_2 (0.5% CO_2; Union Carbide Canada Ltd.). The fungus was grown in unsealed petri dishes placed on a platform in sealed plastic chambers (50 by 30 by 15 cm). These sterile chambers were connected through air filters (0.22-μm pore size; μstar; Costar, Cambridge, Mass.) to the gas cylinders, and they were provided with an air flow of 1 volume/100 min. The floor of these plastic chambers was flooded with 2 liters of sterile water to prevent possible desiccation of the cultures. This experiment was performed twice.

Labeling experiments with ^{14}C isotope. On one side of a round divided petri dish (100 by 15 mm, two compartments; Falcon; Becton Dickinson Labware, Oxnard, Calif.), one root explant was grown on 10 ml of M medium supplemented with 40 μCi of [U-^{14}C]sucrose (350 mCi/mmol; ICN Biomedicals, Inc.) (Fig. 2). On the other side of the plastic division, several spores were germinated on a polycarbonate membrane (Nuclepore Corp., Pleasanton, Calif.) which lay on ME medium under a thin layer of M medium. The petri dish was incubated in the inverted position for 2 weeks. Then, the membrane with adhering agar, spores, and hyphae was removed and rinsed by being laid in several changes of tap water for 2 h. For autoradiography, the material was dried at 60°C for 2 h onto a sheet of glass and autoradiographed on Kodak XAR-5 X-ray film for an exposure of 3 weeks at 25°C. For controls, polycarbonate membranes were first incubated for 2 weeks in the presence of roots growing on radioactive sucrose before being used for germinating spores in the presence of roots growing on nonradioactive sucrose.

Assessment of fungal growth. The linear growth of hyphae in millimeters was recorded as previously described (3a). When contacts between hyphae and roots were permitted, the number of infection units was counted after the roots were cleared in 10% (wt/vol) KOH for 10 min, rinsed in water, and stained in 0.1% (wt/vol) chlorazol black E for 2 h (8). A total of 7 to 15 replicates were performed for each experimental condition.

RESULTS

Stimulation of fungal growth by root factors. After 5 weeks of culture in the bicompartmental system, fungal growth was greatly stimulated (eightfold) by the simultaneous presence of root volatiles and exudates (MEV, Fig. 3). In the absence of root volatiles, fungal growth without and with exudates (M and ME media, respectively) did not differ significantly. The presence of root volatiles improved hyphal elongation on M medium by 130% and that on ME medium by 460%, indicating that root exudates and volatiles acted synergistically. The growth curves show that this synergistic effect increased the initial rate of fungal growth.

Effect of CO_2 on fungal growth. The two experiments gave very similar results. Fungal growth in normal air (0.03%

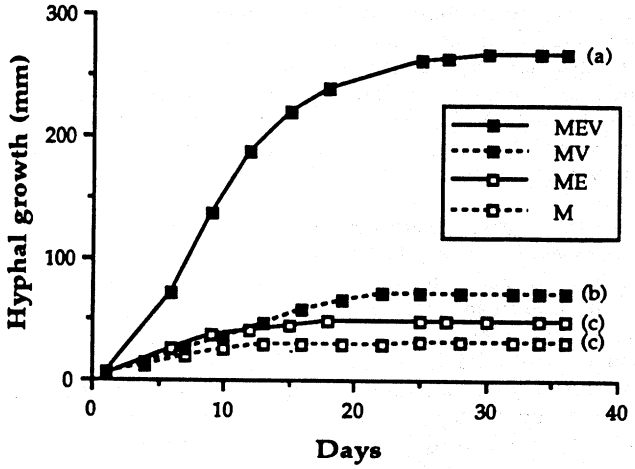

FIG. 3. Hyphal growth from germinating spores of *G. margarita* in the absence of root factors (M medium), the presence of root exudates only (ME medium), the presence of root volatiles only (MV medium), and the presence of both root factors (MEV medium). The curves with different letters have significantly different final values for hyphal growth (Waller-Duncan, $P < 0.05$).

CO_2) was almost nil after 3 weeks on M and ME media, but a great stimulation of growth was observed in 0.5% CO_2, especially on ME medium (Fig. 4). The pH of all four agar media was measured at the end of the experiment with a surface electrode. The media corresponding to the treatments with and without CO_2 supplementation had approximately the same pH (averaging 5.9 and 5.8, respectively). Only a slight difference in pH was observed between media with and without root exudates (averaging 5.7 and 6.0, respectively). Therefore, growth stimulation at 0.5% CO_2 was apparently not due to a change in the pH of the medium.

Effect of trapping CO_2 on fungal growth. In the bicompartmental system, the trapping of CO_2 in root volatiles had a drastic inhibitory effect on fungal growth on ME medium (Fig. 5). The removal of CO_2 immediately and completely stopped hyphal elongation. Analyses of gas samples taken at the end of the experiment confirmed the effectiveness of the KOH traps. No CO_2 was detected in dishes containing KOH traps, whereas 0.5 to 2% CO_2 levels were detected in dishes without traps.

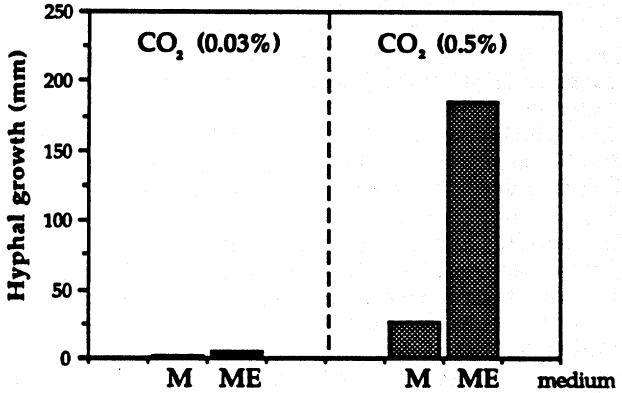

FIG. 4. Hyphal growth from germinating spores of *G. margarita* after 3 weeks in the absence (M medium) and presence (ME medium) of root exudates under two CO_2 concentrations. Growth values are not significantly different for 0.03% CO_2 (Student's t test, $P = 0.28$), but they are significantly different for 0.5% CO_2 (Student's t test, $P = 0.0002$).

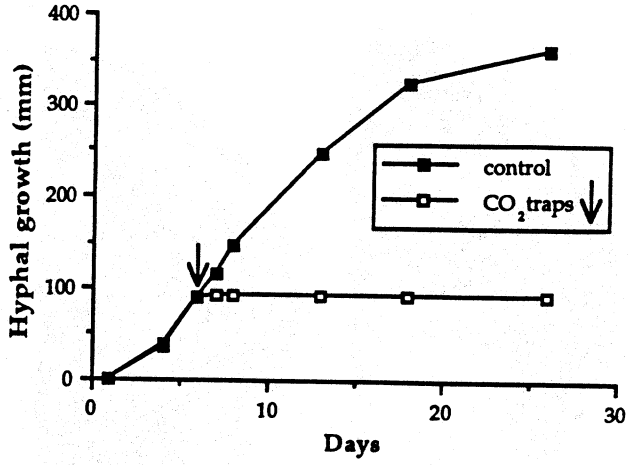

FIG. 5. Hyphal growth from germinating spores of *G. margarita* in the presence of root exudates and volatiles (MEV medium) stops when KOH traps are added (↓).

When root infections were permitted, the state of biotrophy was achieved in all cases since fungal growth continued after the severance of the spore (Fig. 6). Subsequent suppression of CO_2 decreased the growth of the extramatrical phase. The Wilcoxon test was used to compare the final growth obtained in the two treatments, and it indicated that there is a 13% probability that there was no difference between the results. A positive correlation was obtained between the growth of the extramatrical phase and the number of root infections for the control and for the treated dishes, the coefficients of Spearman rank correlation being 0.84 ($P = 0.019$) and 0.90 ($P = 0.006$), respectively. A total of 2 to 10 root infections per petri dish were observed in the control treatment, and 1 to 6 were observed in the treatment with KOH traps, the mean values being 4.4 and 3.1, respectively. The observed difference in fungal growth was apparently not due to a change in root growth because there was no significant difference (Student's t test, $n = 3$, $P = 0.81$) between the fresh weight of roots after 2 weeks of growth with and without the KOH traps (0.44 and 0.43 g, respectively; five root apices initially 15 mm long were used per replicate).

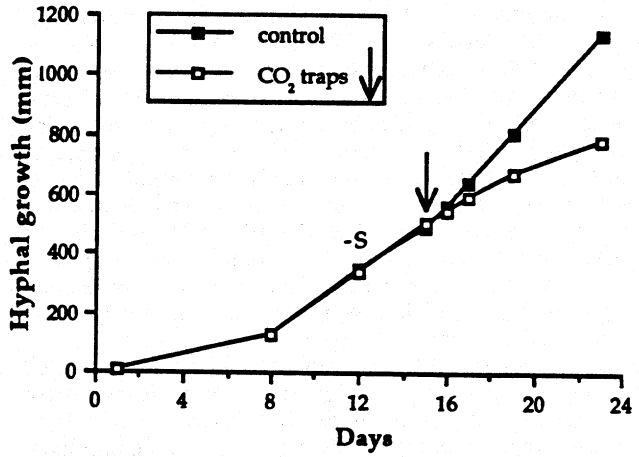

FIG. 6. Extramatrical hyphal growth rate of *G. margarita* after root colonizations and removal of the spore (−S) decreases when KOH traps are added (↓).

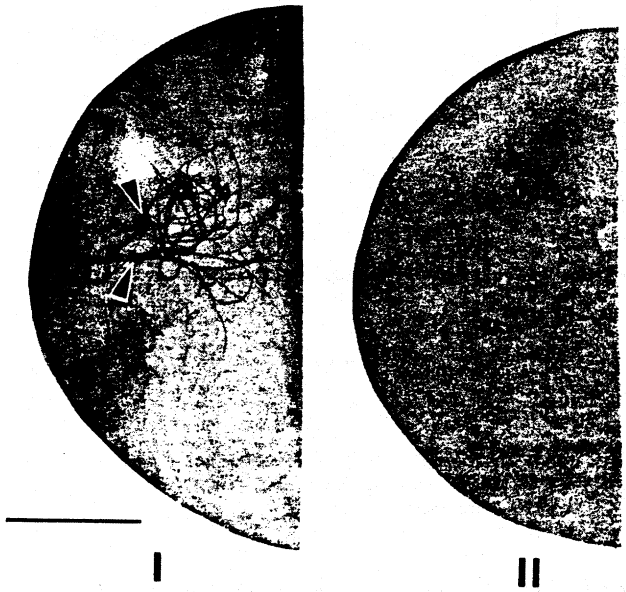

FIG. 7. (I) Autoradiogram showing the fixation of radioactive matter by germinating spores (arrowheads) of *G. margarita* after 2 weeks of incubation on ME medium in one compartment while being exposed to ^{14}C-labeled root volatiles provided by roots growing on [U-^{14}C]sucrose in a second compartment. (II) No radioactive labeling was detected in the fungus in the control treatment. Bar, 2 cm.

Labeling experiment. In the labeling experiment, the fungus was placed under optimal conditions for growth, i.e., on MEV medium. The experimental conditions were designed to provide $^{14}CO_2$ to the fungus. The first autoradiogram (Fig. 7) shows that the spores and the hyphae were labeled by radioactive matter. The spores were found to be labeled only when germination occurred. Despite substantial background radioactivity in the control treatment (second autoradiogram), there was no labeling of the fungus. This suggests that a root volatile(s) solubilized into the agar medium was not taken up by the fungus.

DISCUSSION

Growing roots have been previously reported to stimulate in vitro hyphal growth of certain VAM fungi (3a, 17, 21). This apparently constitutes one of the first interactions between a plant and a VAM fungus during the preinfection stage. Our results suggested that roots stimulate VAM fungi to use their spore reserves by two simultaneous actions: changes in the medium and changes in the composition of the culture gases. Carbon dioxide alone at 0.5% was able to replace root volatile compounds in the activation of fungal growth on spore reserves, resulting in fungal growth that was considerably greater than that at 0.03% CO_2. This result strongly suggests that CO_2 produced during root respiration was the major and determinant change in gas composition owing to the presence of growing roots, despite the fact that they do produce many other volatile compounds.

Even though the two experiments had been conducted independently, two interesting patterns could be seen when comparing fungal growth in the bicompartmental culture system and that under artificially controlled CO_2 conditions. First, fungal growth on M and ME media in the absence of root volatiles (Fig. 3) was greater than that obtained under a stream of normal air (Fig. 4). Decreased growth in the latter case may be attributed to the continuous air stream which prevented accumulation (up to 0.1%; data not shown) of the CO_2 produced by the fungus itself. Second, fungal growth in the presence of root volatiles (Fig. 3) was greater than that found with 0.5% CO_2 (Fig. 4). The possible explanations are that the CO_2 level was not optimal or that there are additional root volatile factors which stimulate fungal growth, or both.

Nevertheless, experiments employing KOH to trap CO_2 confirmed the essential role of CO_2. Similar experiments had been performed (data not shown) with traps saturated with $KMnO_4$. Permanganate is a strong oxidant of many organic compounds (28), including volatile ketones and aldehydes, which can also be neutralized by KOH (13). The observation that $KMnO_4$ did not suppress the stimulatory effect of root volatiles on fungal growth reinforces the view that KOH acted by suppressing CO_2. This is the first mention that CO_2 is a key stimulatory factor for the growth of a VAM fungus. Le Tacon et al. (20) have previously reported that 5% CO_2 detrimentally and irreversibly affected in vitro growth of *Glomus mosseae*. This apparent contradiction to our results is likely due to the 10-fold difference in CO_2 concentrations.

Carbon dioxide is well-known to have an effect on the metabolism and growth of fungi (15, 30). Some fungi can be tolerant to high concentrations of CO_2 and be stimulated in their mycelial growth. The optimum concentrations of CO_2, as well as inhibition thresholds, depend on the fungi and on the growth conditions. Zadrazil (32) demonstrated that growth of some *Pleurotus* species is stimulated by high CO_2 concentrations ranging up to 28.8%, whereas San Antonio and Thomas (26) showed that CO_2 concentrations greater than 0.1% are inhibitory for *Agaricus bisporus*. CO_2 has also been shown to be a limiting factor for the growth of biotrophic fungi. Boasson and Shaw (6) reported that 1% CO_2 is essential for colony initiation by flax rust fungus grown in vitro. Straasma et al. (29) demonstrated that CO_2 is a key factor in shortening the lag phase of growth for the ectomycorrhizal fungus *Cantharellus cibarius* Fr. Carr et al. (9) used suspension-cultured plant cells to show the involvement of a volatile substance in the improvement of hyphal growth in the VAM fungus *Glomus caledonium*. Their experimental protocol, in our opinion, does not allow the rejection of the hypothesis that CO_2 is involved. Finally, the germ tube of *Gigaspora gigantea* is also affected by root volatile compounds (13, 19). The chemotropism of aerial germ tubes toward roots is inhibited in the presence of either $KMnO_4$ or KOH. In light of our findings, it would not be surprising that the removal of CO_2 inhibits the chemotropic attraction at least by the indirect action of inhibiting germ tube growth.

The absolute CO_2 dependence of hyphal growth from *G. margarita* spores is not surprising, considering that most carbon reserves in the spores of VAM fungi are in form of lipids (4, 5, 18). The catabolism of lipids yields only acetyl coenzyme A (a C_2 molecule) as an input to the Krebs cycle. This cycle expends two carbons (in the form of CO_2) in each turn, so that the net balance in carbon is zero. The intermediates of the Krebs cycle are the only links between the catabolism of lipids and the anabolism of other organic compounds. Unless there is an alternative carbon input, anabolism cannot proceed. The anaplerotic pathway which fixes CO_2 therefore becomes an essential source of carbon in germinating spores of VAM fungi. According to this hypothesis, the labeling of the fungus in the presence of radioactive volatiles with $^{14}CO_2$ is expected.

Bécard and Piché (3a) proposed that when *G. margarita*

switched from the preinfection state (M1 mechanism) to the biotrophic state (M2 mechanism), induction factors from roots may still be required for fungal growth despite the switch in nutritional source. The inductive functions of CO_2 in M2 were examined with KOH traps. The KOH traps reduced fungal growth to a certain extent but did not stop growth. It remains possible that CO_2 is essential for the catabolism of lipids along extramatrical hyphae, where they are likely the preferred form of storage and transport carbon during the symbiosis (10). The limited effect of the KOH traps may be explained by the ability of the traps to remove only the excess CO_2 which had escaped both symbionts. However, it is also possible that alternative carbon sources, other than lipids, supplied by the host roots were rapidly used for fungal growth.

The importance of CO_2 during symbiosis had also been examined by Saif (24). In three host plants grown on soil maintained at 16% O_2, the percentage of root length infected with *Glomus macrocarpum* and the number of vesicles in the roots were greater at 0.5% CO_2 than at 0%. The optimum CO_2 concentration depended on the plant host, ranging from 2 to 4%. Concentrations of CO_2 ranging from 0.5 to 1% also stimulated growth of the plants, especially when they were mycorrhizal. Saif (24) proposed that the greater sensitivity of the mycorrhizal plants was due to an indirect positive effect of CO_2 on the extraradical phase of the fungus. These positive effects of low CO_2 concentrations are consistent with our expectations when we consider the stimulatory effects of 0.5% CO_2 suggested in our study.

Many studies have been conducted to determine the influence of plant root exudates on VAM colonization (2, 12, 14). In general, exudates do not improve spore germination but can stimulate fungal growth or VAM colonization rate or both. Some studies have shown a correlation between the quantity of soluble root carbohydrates and the improvement of fungal growth (1, 25). It has been suggested that phosphorus deficiency increases root membrane permeability, which results in increased net loss of metabolites and therefore increased VAM infections (14). However, other studies did not find any relationship between the total sugar content in root exudates and the degree of VAM infection under axenic conditions (2). Elias and Safir (11) suggested that it is the quality of the exudates from plants experiencing P deprivation which is important. Despite the various contradictions, it remains clear that root exudates can greatly influence the growth of VAM fungi. The active components remain to be determined. Some of them could be specific root metabolites which induce fungal growth according to the hypothesis of plant-produced derepressors as proposed by Brian (7). In some plant-microbe interactions, this hypothesis has been largely confirmed and root signals have been well determined. The examples include acetosyringone molecules, which induce specific virulence genes in *Agrobacterium tumefaciens*, and flavones, which induce nodulation genes in *Rhizobium* species (22, 23, 27). These interactions between plant host and bacteria constitute possible models for interpreting some of our results.

Finally, it must be emphasized that the synergy between CO_2 and other root factors in inducing fungal growth is a very interesting feature. From an ecological point of view, this synergy is consistent with the idea that a spore of *G. margarita* cannot exhaust its reserves in the presence of CO_2 produced by soil microflora unless it is also in the proximity of living roots. From a technological point of view, this synergy may constitute a valuable clue about the required conditions for the pure culture of VAM fungi.

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